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13. ABSTRACT ***(Maximum 200 Words)***

Amplification of the epidermal growth factor receptor (EGF-R) in breast cancer portends a poor prognosis. We utilized a cell culture model based on the MDA-MB-468 (468) cell line to address the mechanism by which receptor amplification can increase receptor activation in cancer and provide a cellular growth advantage. We show that amplification of the EGF-R is accompanied by a corresponding increase in the number of activated EGF-R per cell. This increased level of EGF-R activation is autocrine ligand dependent and is associated with an increased sensitivity to autocrine loop interruption. However, the increased receptor activity was not due to an elevated level of autocrine ligand production. We found that the EGF-R negative regulatory controls including internalization, downregulation, desensitization and phosphatase activity did not become limiting following receptor amplification. In contrast, we show that receptor amplification increases the ability of a cell to capture autocrine ligand. This increase in capture efficiency can account for the increased level of activation. Although the increased EGF-R activity following EGF-R amplification is ligand dependent, we also show that amplification of Her2 may increase the level of EGF-R activation in a ligand independent fashion. This would suggest that Her2 amplification promotes increased activity by some other mechanism.

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Kim Shook 6/29/00

PI - Signature Date

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Introduction

Amplification of the epidermal growth factor receptor (EGF-R) is closely correlated with the carcinogenic phenotype *in vitro* and *in vivo* (1, 7, 8, 14, 18, 24, 26, 28, 33, 36). More importantly, amplification of the EGF-R portends a poor prognosis for women diagnosed with breast cancer (19, 20, 27). The EGF-R is a membrane bound receptor tyrosine kinase (5, 12, 13, 35). Binding of any one of the receptors six naturally occurring ligands activates the receptors intrinsic tyrosine kinase (15, 22, 23, 29, 31, 32, 34). The activated kinase then autophosphorylates tyrosine residues on the receptors COOH terminal tail (15, 22, 23, 29, 31, 32, 34). The phosphorylated tyrosines provide docking sites for SH2 containing proteins such as Grb2, which promote signal transduction through other cellular proteins such as Ras (4, 21). The mechanism by which amplification of the receptor promotes increased receptor activity, and thereby leads to unrestrained growth of a cancer cell, was the subject of this pre-doctoral grant. We found that amplification of the receptor appears to increase the cells ability to capture autocrine ligand. Our originally proposed hypothesis, which suggested that receptor amplification decreased the cells ability to negatively regulate receptor activity, was proven incorrect.

Body

At the initiation of this grant, we proposed that amplification of the EGF-R, without concomitant amplification of its negative regulatory apparatus, would increase its activity and promote aberrant signaling. In order to test this hypothesis we utilized a system based on the malignant breast cancer cell line MDA-MB-468 (468) (10, 11). The 468 cell line expresses 10^6 EGF-R per cell due to a chromosomal amplification. Filmus *et al*, isolated "S" variants of the 468 cell which have lost the chromosomal amplification and now only express approximately 10^4 EGF-R per cell (10, 11). We then further increased the utility of the system by amplifying the receptor numbers in the S variants by retroviral mediated transduction to generate "XR" cell lines expressing approximately 10^5 EGF-R per cell. In our 1997 Annual report, we showed that, following treatment with EGF, receptors in the 468 cell line remained activated for a longer period of time than receptors in the S variants (Figure 1). As expected, EGF-R in the XR lines remained active for an intermediate amount of time (Figure 1). This result was consistent with our original hypothesis.

The first part of our proposed Statement of Work was as follows: "Determine the percent reduction of EGF-R kinase activity following PMA or EGF induced desensitization for all cell types being analyzed." Preliminary experiments (data not shown) suggested that we could not observe a desensitization effect in response to EGF. Since this was a negative result, we could make no conclusions with regard to this experiment and it was no longer pursued. As stated in the 1997 annual report, we also attempted to characterize the degree of receptor desensitization in our cells in response to the phorbol ester PMA. As is shown in figure 2, we observed no difference in the phosphotyrosine response in any of our cells in response to PMA. Therefore,

we concluded that desensitization of the EGF-R in our cells by phosphorylation at T654 was not a relevant negative regulatory phenomenon.

Because desensitization of the EGF-R in our cells was not an assayable phenomenon, we sought to compare other EGF-R negative regulatory phenomena to determine if they had become limiting following receptor amplification. As stated in the 1997 annual report, we first looked and the ability of cells to regulate the EGF-R by ligand induced downregulation (Figure 3). As is shown in figure 3B, the percent downregulation of EGF-R in amplified cells is attenuated following receptor amplification. However, the absolute number of EGF-R downregulated (calculated by multiplying the percent downregulated over time by the initial receptor number) was similar, and possibly faster, in amplified lines compared to unamplified lines. This result suggested that downregulation does not become a limiting regulatory mechanism following receptor amplification at physiological (low occupancy) ligand concentrations.

We had performed a PY ELISA normalized for EGF-R using our cells as previously described (30). As shown in figure 4, 50% of the PY is eliminated from S variant cell EGF-R within 1 hour. In contrast, the 468 line still had almost 100% of its PY at the same time point. Since PY/EGF-R will only decrease when PY is removed faster than the EGF-R is degraded, this data suggested that EGF-R in unamplified lines were dephosphorylated before downregulation. However, PY associated with the EGF-R in amplified cell lines was primarily eliminated by downregulation.

One possible explanation for the data shown in figure 4 was that phosphatases had become limiting following receptor amplification. In figure 5, as was shown in the 1999 Annual Report, we show that the phosphatase activity is not limiting following receptor amplification. In fact, EGF-R in the amplified cell lines, paradoxically, appear to be dephosphorylated faster than

receptors in unamplified cell lines. We believe this is due to competition between tyrosine phosphatase access and SH2 binding protein access to the activated EGF-R (2). In this model, amplification of the EGF-R causes SH2 containing proteins to become limiting. Therefore, there is little, or no, competition between these proteins and the phosphatases for the activated EGF-R and the apparent rate of dephosphorylation is faster. Regardless of the final mechanism, phosphatase activity is not limiting.

The second goal proposed in our original Statement of Work was as follows: "Transfect all cell types with mutant receptors and characterize the total receptor number for each transfectant." As stated above, wild type EGF-R were transduced into the S variant lines using retrovirus in order to generate the XR lines. The XR lines express approximately 10^5 EGF-R per cell due to this transduction. However, as stated in the 1997 Annual Report, the 1998 Annual Report and in data presented above, because desensitization of the EGF-R was not observed in response to either EGF or PMA, we did not feel that receptor regulation at S1046/1047 or T654 were relevant to our cell types. Therefore, receptors mutant at these residues were not constructed.

We did address one more potential negative regulatory process in the 1998 Annual Report to determine if it had become limiting following receptor amplification. As is shown in figure 6, the percent EGF-R internalized over time does decrease. However, as was stated for the downregulation data shown in figure 3, the absolute number of EGF-R internalized over time (calculated by multiplying the percent downregulated over time by the initial receptor number) was similar, and possibly faster, in amplified lines compared to unamplified lines. Again, this would suggest that receptor internalization is not limiting at physiological (low occupancy) ligand levels.

The remaining parts of the proposed Statement of Work were as follows: A) "Determine the percent reduction in tyrosine kinase activity following desensitization with either PMA or EGF for all transfected cell types." and B) "Determine the mitogenic response of all transfected cell types." Obviously, both of these proposed research directions are dependent on the receptors being desensitized in response to EGF or PMA. Since this was not the case, we altered our Statement of Work in the 1999 Annual Report.

> **Part IV A)** Show that EGF-R ligand half lives are prolonged in the amplified cell lines.
> Months 37-40
>
> **Part IV B)** Show that basal phosphotyrosine increases following EGF-R amplification are associated with an increase in receptor signaling complexes formed.
> Months 41-44
>
> **Part IV C)** Show that EGF-R basal phosphotyrosine increases in a ligand dependent manner following EGF-R amplification but in a ligand independent manner following Her2 amplification.
> Months 45-48

We had made several conclusions regarding activation of the EGF-R following amplification in the 1999 Annual Report. First, as shown in figure 7, receptor amplification promotes a proportional increase in activated EGF-R in the absence of exogenous ligand. This activation is dependent on autocrine ligand since, as shown in figure 8, addition of the antagonistic antibody 225 and Batimastat, which blocks release of autocrine ligand, blocks most of the phosphotyrosine activity. Figure 9 shows that addition of the antagonistic antibody 225 also inhibits the growth of amplified receptor containing cells better than the unamplified cells. In other words, amplification makes the cells hypersensitive to autocrine loop interruption. Lastly, figure 10 shows that, although autocrine ligand production for the 468 line may be slightly higher than the S variants, at most it is two fold. This increase cannot explain the 20 fold increase in PY observed in figure 7 following receptor amplification.

Data presented to this point suggests the following: First, receptor amplification is associated with increased receptor activity. Second, this activity is dependent on autocrine ligand production from the cells. Third, the increased activity is not due to an increase in the concentration of ligand produced by the cells. Fourth, receptor amplification makes the cells hypersensitive to autocrine loop interruption. Fifth, negative regulatory processes such as phosphatase activity, internalization rate, downregulation rate and desensitization do not become limiting following receptor amplification.

How can all of these points be combined into a single testable hypothesis? As receptor density increases, the likelihood that an autocrine ligand molecule (after release) will encounter an empty receptor before being lost to the media also increases. Thus, although the cells produce similar amounts of ligand, the cell with a higher receptor number would be more likely to capture that ligand. If the receptor amplified cell lines capture more ligand, there will be more activated EGF-R per cell and the level of PY will be increased. This "capture efficiency" hypothesis makes a specific prediction. For any concentration of ligand, that ligand is more likely to be lost to the media in unamplified cell lines than in receptor amplified cell lines. In simpler terms, ligand will remain associated with cells containing higher receptor numbers for a longer period of time than in cells with low receptor numbers.

A test of the "capture efficiency" hypothesis was outlined in the 1999 Annual Report Statement of Work: "Show that EGF-R ligand half lives are prolonged in the amplified cell lines". Figure 11 shows that this is indeed the case. Cells were pulsed for 10 minutes with radiolabeled ligand. The radioactive media was then changed and the amount of radioactivity associated with the cell from 0-30 minutes post chase was determined by soluablization in SDS. Times of under 30 minutes were required because, after 30 minutes, ligand degradation in the

lysosome, becomes significant and prevents accurate measure of ligand loss only to dissociation. If cells at 0 minutes post chase are arbitrarily defined as having 100% of the ligand, the time at which only 50% of the ligand remains is the half life of ligand association with the cell. Curves were fit to the data points obtained from 0-30 minutes post chase and the half life of ligand in each cell type was extrapolated by extension of that line. Figure 11 shows that the half life of ligand association with the cell does indeed increase with increasing receptor number as predicted by our hypothesis.

The second part of the revised Statement of Work we proposed in the 1999 Annual Report was as follows: "Show that basal phosphotyrosine increases following EGF-R

amplification are associated with an increase in receptor signaling complexes formed." Although we tried for several months, we were never able to observe an increase in the number of signaling complexes formed following receptor amplification. Potential signaling partners tried included Grb2, SOS, Activated MAPK, Eps 8, and c-Cbl. Our failure to observe any increased signaling complexes represents a negative result and we can therefore make no conclusions regarding this result. However, it is our opinion that the problem is due to detection limits and not necessarily to a lack of signaling complexes. In addition, since we know the growth of the cells is increased following receptor amplification, at least some signaling pathways are increased in intensity even if we could not observe it.

The last part of the 1999 Annual Report revised Statement of Work is as follows: "Show that EGF-R basal phosphotyrosine increases in a ligand dependent manner following EGF-R amplification but in a ligand independent manner following Her2 amplification.". Figure 8 and figure 9 demonstrate that the observed PY increases, following EGF-R amplification, are ligand dependent processes. In contrast, figure 12 shows that increases in phosphotyrosine associated

with EGF-R following Her2 amplification are ligand independent. The MTSV cell line expresses a small number of Her2 receptors and its EGF-R associated PY is only partially inhibitable by antagonistic antibodies. Amplification of Her2 in MTSV cells by transfection was used to generate the CE2 line. As is also seen in figure 12, the EGF-R associated PY in those lines is inhibited even less than seen for the MTSV line following treatment with antagonistic antibodies. Thus it appears that EGF-R basal phosphotyrosine increases in a ligand dependent manner following EGF-R amplification but in a ligand independent manner following Her2 amplification.

Key Research Accomplishments

- EGF-R amplification is associated with increased receptor activity.
- The increased activity associated with the EGF-R following receptor amplification is dependent on autocrine ligand production from the cells.
- The increased EGF-R associated activity is not due to an increase in the concentration of autocrine ligand produced by the cells.
- EGF-R amplification makes the cells hypersensitive to autocrine loop interruption.
- Negative regulatory processes such as phosphatase activity, internalization rate, downregulation rate and desensitization do not become limiting following EGF-R amplification.
- EGF-R amplification increases the ability of cells to capture autocrine ligand.
- Although the increased EGF-R activity following EGF-R amplification appears to be ligand dependent, the increased EGF-R activity which follows Her2 amplification may be ligand independent.

Reportable Outcomes

1) Publication: Schooler, K. and Wiley, H.S. "Ratiometric Assay of Epidermal Growth Factor Receptor Tyrosine Kinase Activation." *Analytical Biochemistry.* **277**:135-142. 2000.

2) Ph.D. in Experimental Pathology successfully defended June 21, 2000.

3) Thesis entitled "Mechanisms For Increasing Epidermal Growth Factor Receptor Activity Following Receptor Amplification." submitted to University of Utah for review and eventual publication.

4) Manuscript in preparation: "Amplification of the EGF-R increases the cells ability to capture autocrine ligand."

5) Manuscript in preparation: "Amplification of the EGF-R prolongs and increases the level of EGF-R activation by activation of internal receptors."

6) Manuscript in preparation: "EGF-R amplification in cancer: A review."

Conclusions

During the course of this grant we were able to demonstrate that EGF-R amplification leads to an increase in EGF-R activity by increasing the cells ability to utilize its own autocrine ligand. Although it has long been known that amplification of the EGF-R is associated with carcinogenesis, the mechanism regarding how amplification of the EGF-R leads to an increase in its activity is not known. In our original hypothesis we suggested that amplification of the EGF-R without concomitant amplification of its negative regulatory components would lead to aberrant receptor activity. However, at least with regards to the negative regulatory processes of internalization, downregulation, desensitization and dephosphorylation, this does not appear to be the case. We tested an alternate hypothesis, where we proposed that receptor amplification could improve the cells ability to capture their own ligand. In this model, ligand expression could remain similar in all cell types and a change in receptor number would lead directly to an increase in the level of receptor activity. As described above, our data is consistent with this hypothesis.

We demonstrated that EGF-R amplification increased the cells sensitivity to inhibition of the cells autocrine loop (figure 9). This result may help explain the low toxicity observed when this antibody is used in the clinic (6, 16, 17). In addition, as shown in figure 8, as receptor levels increase, the ability of either the antagonistic antibody 225 or Batimastat to block constitutive EGF-R activity decreases. However, combining the two treatments results in a synergistic inhibition of this activity. Batimastat has been previously shown to decrease the growth of cells containing EGF-R and it is currently in clinical trials as an anti-metastasis agent (3, 9, 25). Our data suggests that combination chemotherapy using both Batimastat and antagonistic antibodies may provide a much more efficacious alternative to using either compound alone.

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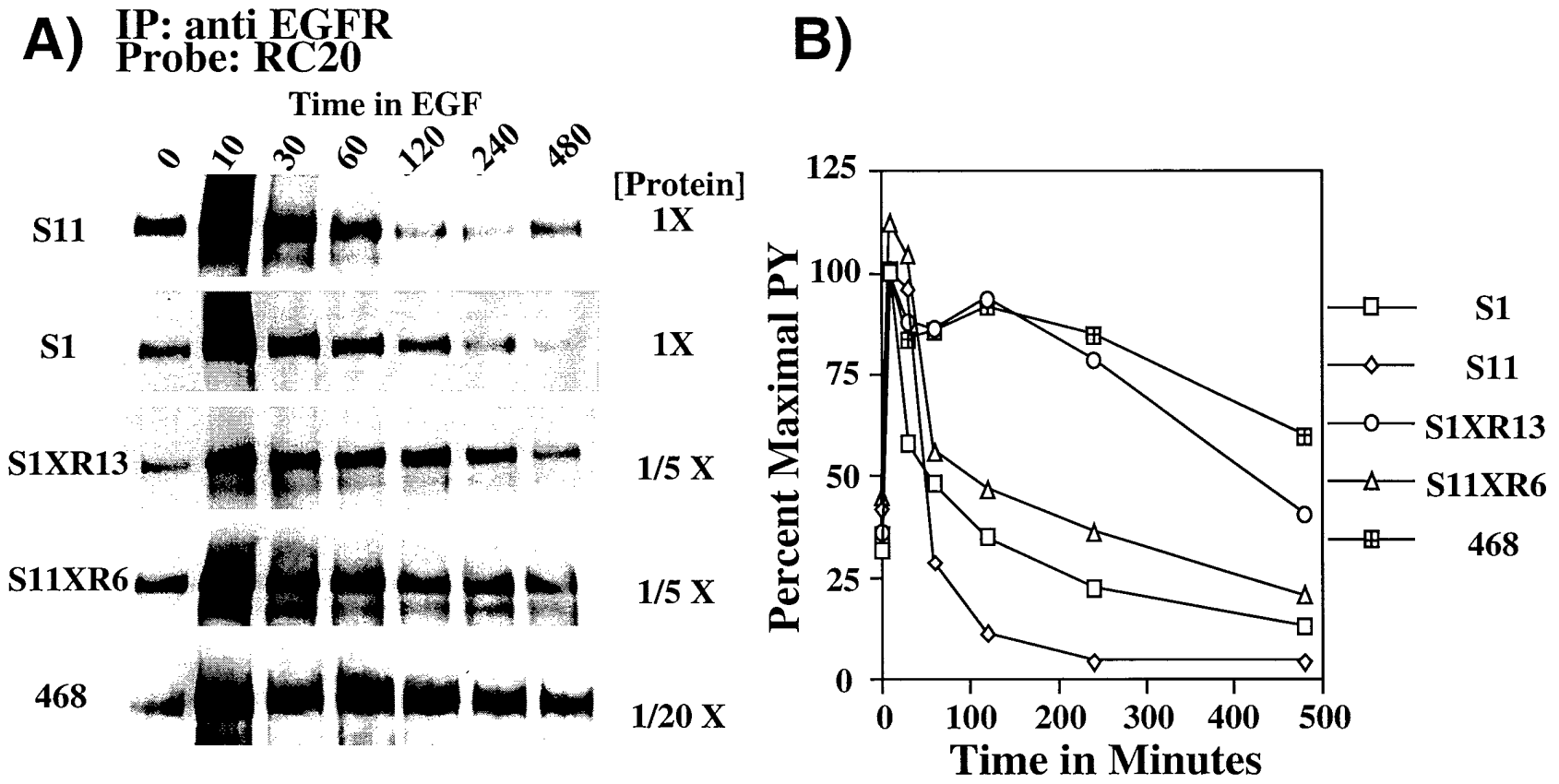


Figure 1: *Ligand induced EGF-R associated PY is prolonged in cell lines which overexpress the receptor.* S1, S11, S1XR13, S11XR6, and MDA-MB-468 were treated with 100 ng/ml EGF for 0, 10, 30, 60, 120, 240 or 480 minutes at 37 C. Cellular protein concentrations were adjusted so that S1 and S11 were 5 times more concentrated than the XR lines and 20 times more concentrated than the MDA-MB-468 cell extracts. The EGF-R was immunoprecipitated with anti-EGF-R antibodies and resolved by SDS-PAGE. The receptor PY was then visualized by chemiluminesence using the HRP conjugated anti-PY antibody RC20 on Western blots. **A)** Western blot of PY associated with the EGF-R. **B)** Densitometric quantification of the blot shown in "A". PY level at 10 minutes is defined as 100%.

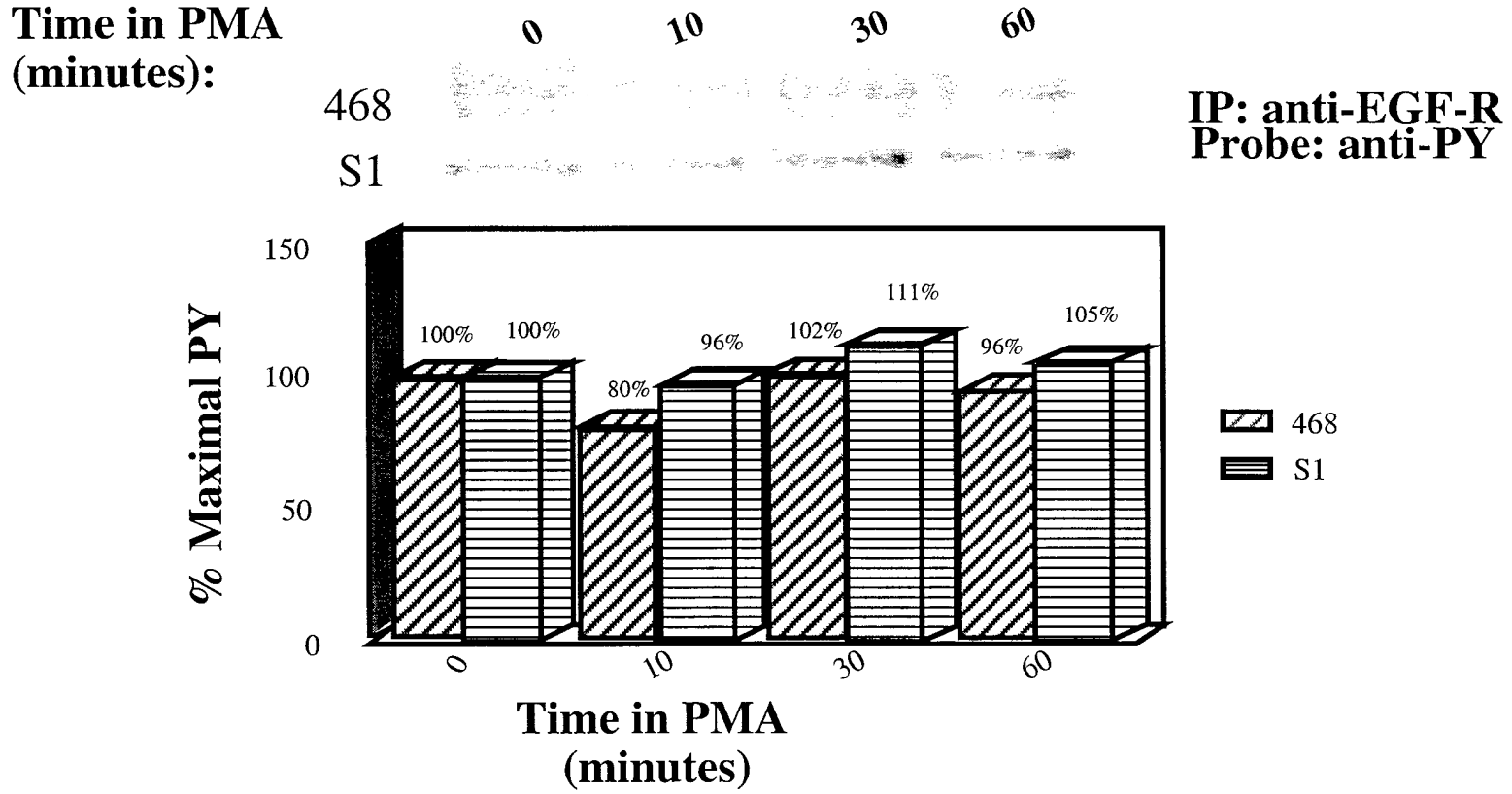


Figure 2: *PMA treatment does not desensitize the EGF-R in either the EGF-R amplified cell line (468) or the non-amplified variant cell line (S1).* Cells were treated for 0, 10, 30 or 60 minutes with 1.6 μM PMA and then treated for 10 minutes with 100 ng/ml EGF. Cellular proteins were extracted and the EGF- R were immunoprecipitated with anti-EGF-R antibody. PY levels were visualized by PAGE followed by western blotting with anti-PY antibodies. Values were normalized to 100% at 0 minutes treatment with PMA.

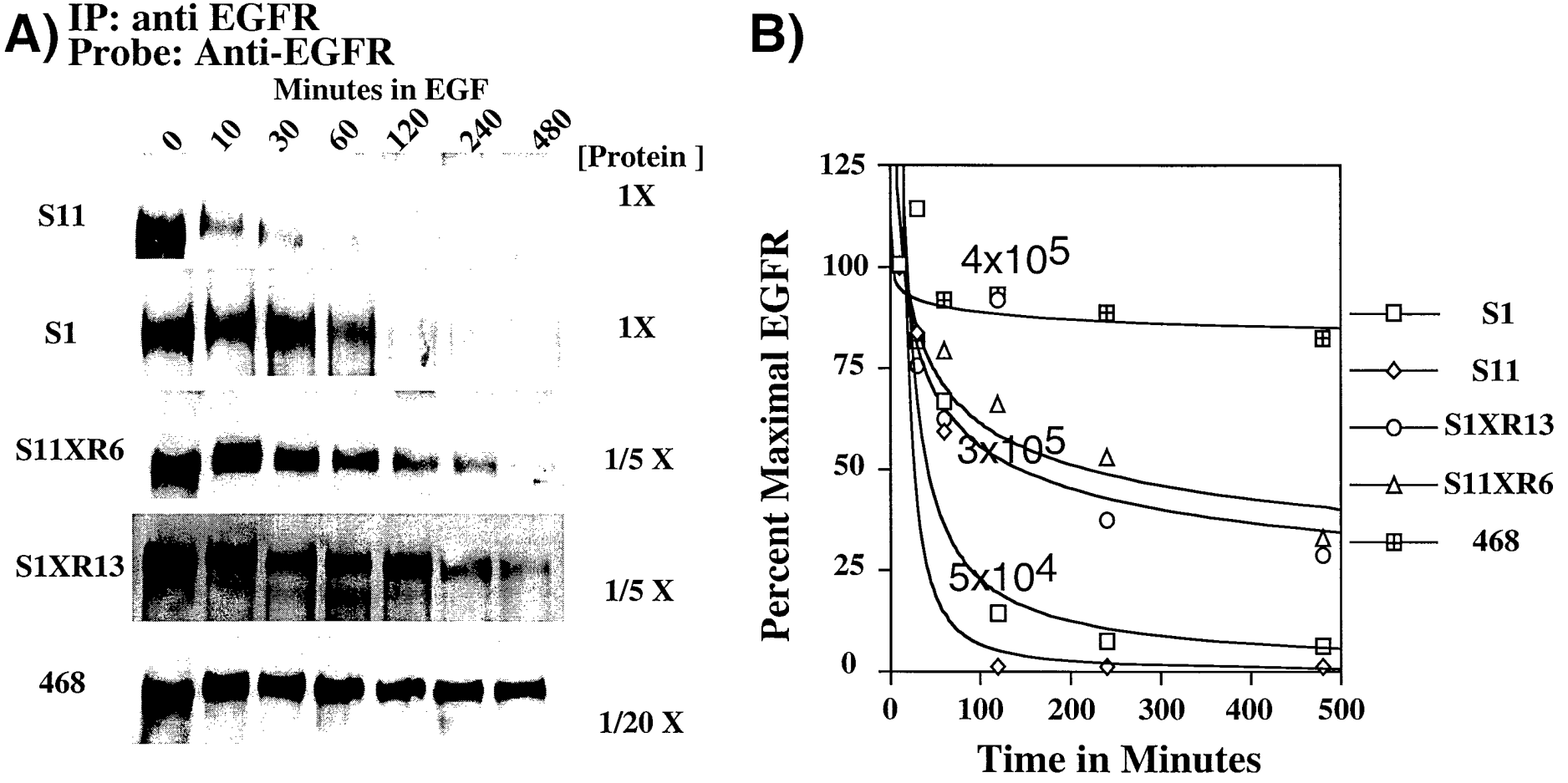


Figure 3: *Rate of EGF-R downregulation is closely correlated with the duration of EGF-R associated PY.* S1, S11, S1XR13, S11XR6, and MDA-MB-468 were treated with 100 ng/ml EGF for 0, 10, 30, 60, 120, 240 or 480 minutes at 37 C. Cellular protein concentrations were adjusted so that S1 and S11 were 5 times more concentrated than the XR lines and 20 times more concentrated than the MDA-MB-468 cell extracts. The EGF-R was immunoprecipitated with anti-EGF-R antibodies and resolved by SDS-PAGE. The receptor EGF-R level was then visualized by chemiluminesence using the primary anti-EGF-R antibody SC03 and an HRP conjugated secondary antibody on Western blots. **A)** Western blot of EGF-R levels. **B)** Densitometric quantification of the blot shown in "A". EGF-R level at 10 minutes is defined as 100%. Exponentiated numbers represent the number of downregulated EGF-R at 60 minutes.

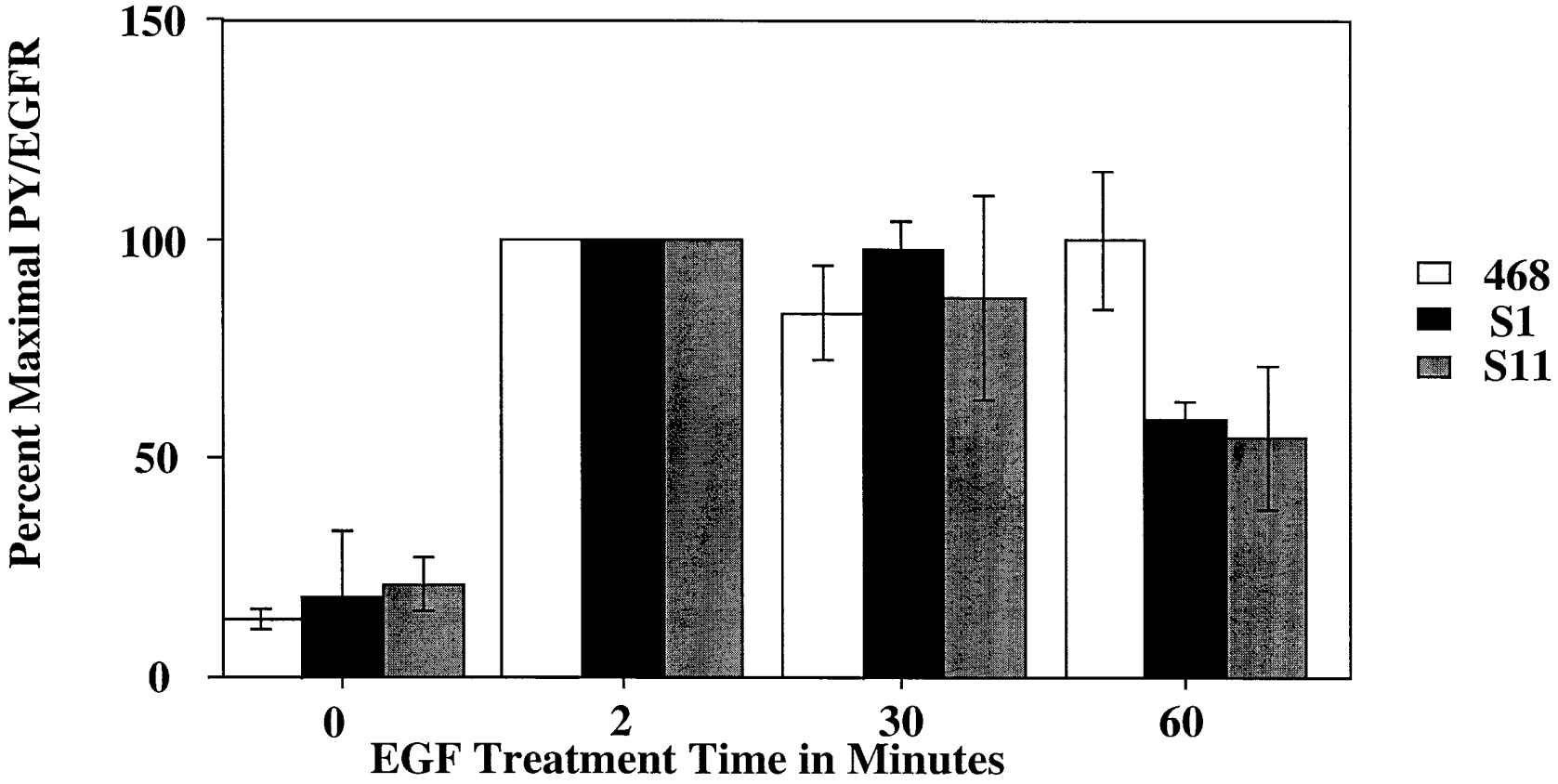


Figure 4: *PY is eliminated faster than the EGF-R is degraded in unamplified lines but is removed at a rate similar to receptor degradation in amplified lines.* S1, S11 and MDA-MB-468 cells were treated with 100 ng/ml of EGF for 0, 2, 30 or 60 minutes at 37 C. PY/EGF-R was determined by parallel PY and EGF-R ELISA assays. The PY/EGF-R level at 2 minutes was defined as 100%.

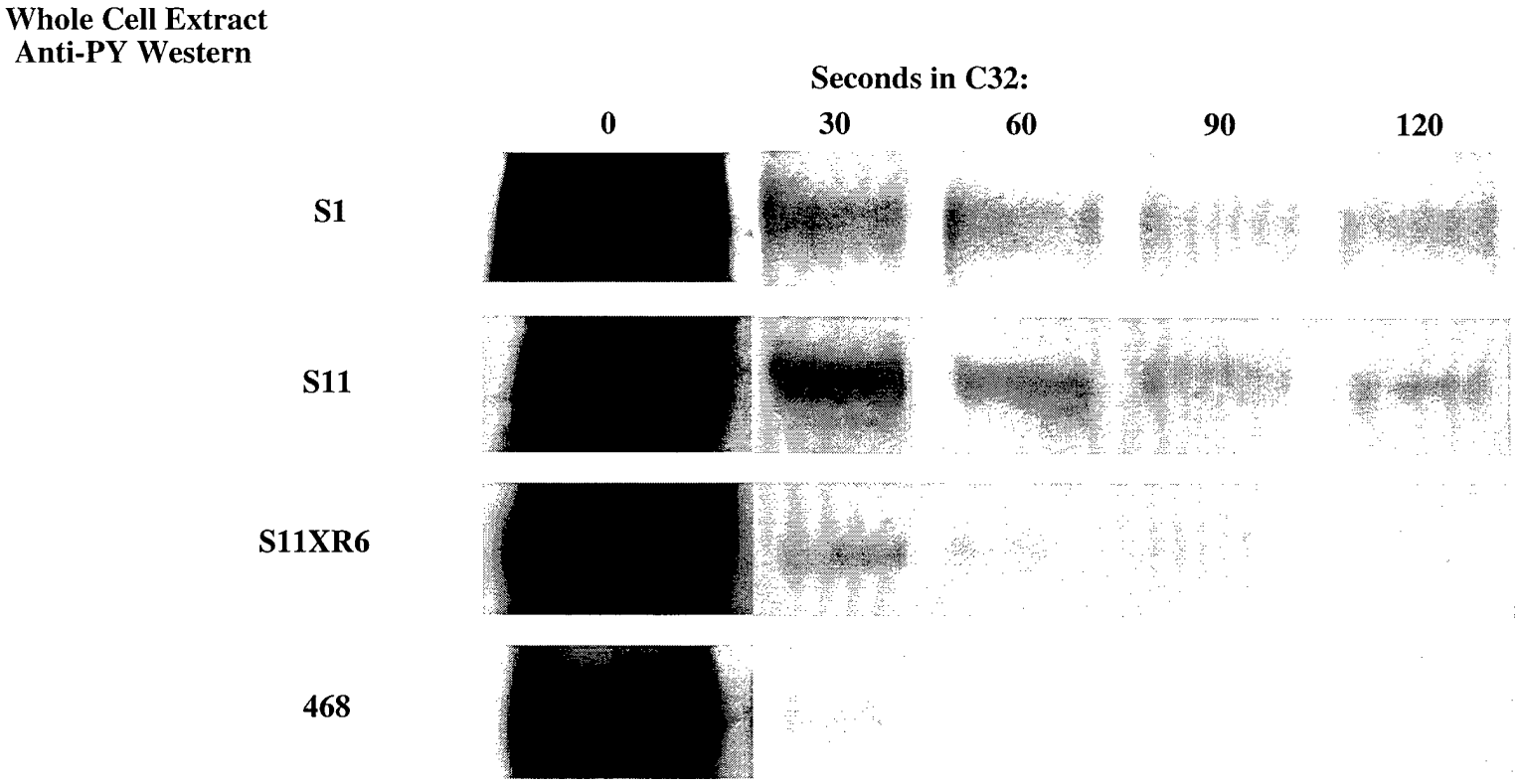


Figure 5: *Tyrosine phosphatase activity is not limiting in receptor amplified cell lines.* S1, S11, S11XR6, and MDA-MB-468 cells were treated with 100 ng/ml EGF for 10 minutes at 37 C. EGF containing media was aspirated and replaced with media containing 4 mM PD 153035 for 0, 30, 60, 90 or 120 seconds to block EGF-R kinase activity and promote receptor dephosphorylation. Dephosphorylation was stopped by aspirating PD 153035 containing media and replacing it with 4 C PBS containing 1 mM orthovanadate. Whole cell extract proteins were resolved by SDS-PAGE and PY levels were visualized by Western blotting and chemiluminesence using the HRP conjugated anti-PY antibody RC20.

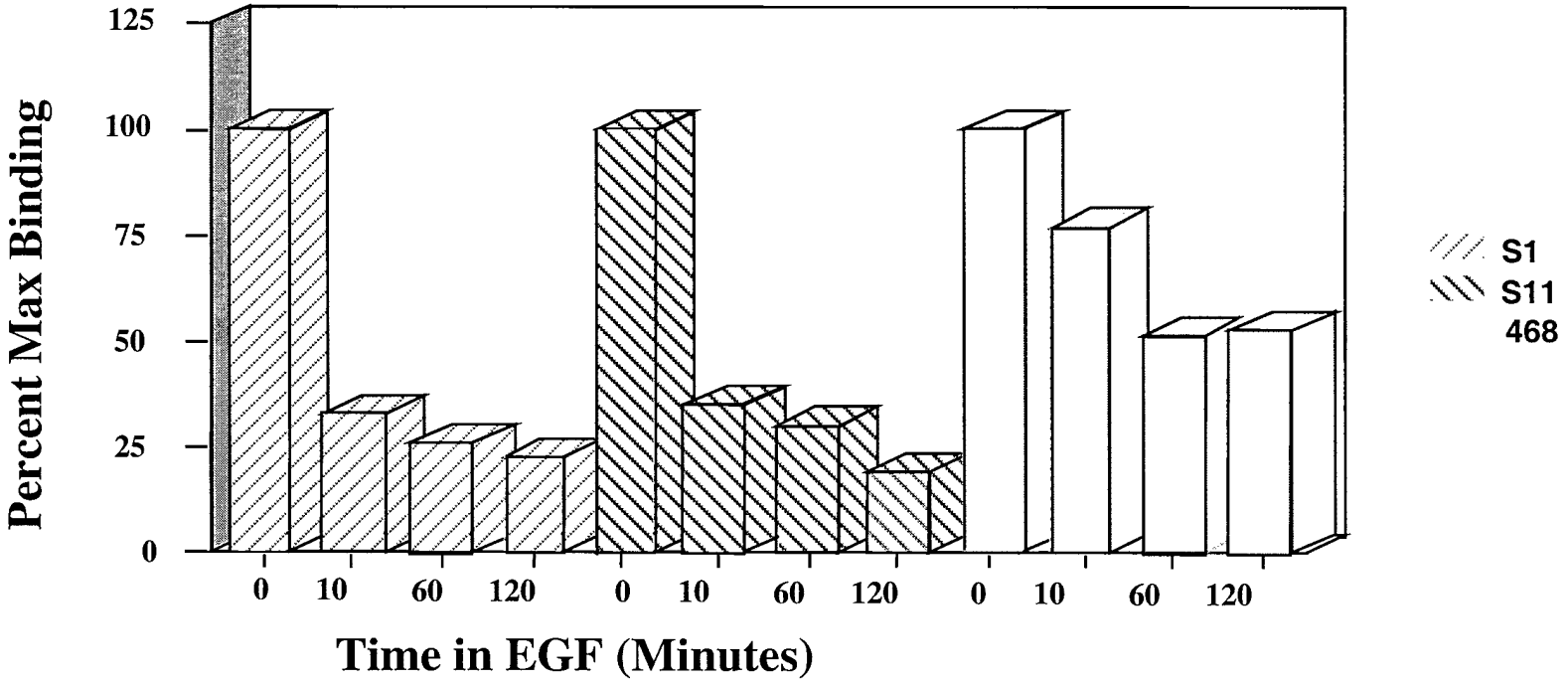


Figure 6: *Activated EGF-R is rapidly removed from the surface of S1 and S11 variants but not from the surface of the parental 468 cell line.* 100 ng/ml EGF was added to S1, S11 or 468 cell media for 0-120 minutes at 37C. After incubation, the cells were placed on ice and washed 3X with ice cold WHIPS pH 7.4 (0.1% Polyvinylpyrolidone, 130 mM NaCl, 5 mM KCl, 0.5 mM MgCl2-6H20, 1 mM CaCl2-2H20, 20 mM HEPES (Free acid)). 1 ml of 3 ug/ml I125 labeled anti-EGFR antibody 13A9 was added to each plate. Plates were then incubated for 2 hours at 4 C. After incubation, plates were again washed 3 times with ice cold WHIPS. 1 ml ice cold glycine/urea strip pH 3.0 (50 mM glycine, 100 mM NaCl, 1 mg/ml Polyvinylpyrolidone, 2 M Urea) was added to each plate and allowed to incubate for 5 minutes and then collected into a gamma tube. Plates were then rinsed once more with another 1 ml of glycine/urea strip and this wash was also collected into the gamma tube. All samples were counted in a gamma counter. Cell number was determine by counting in a Coulter counter.

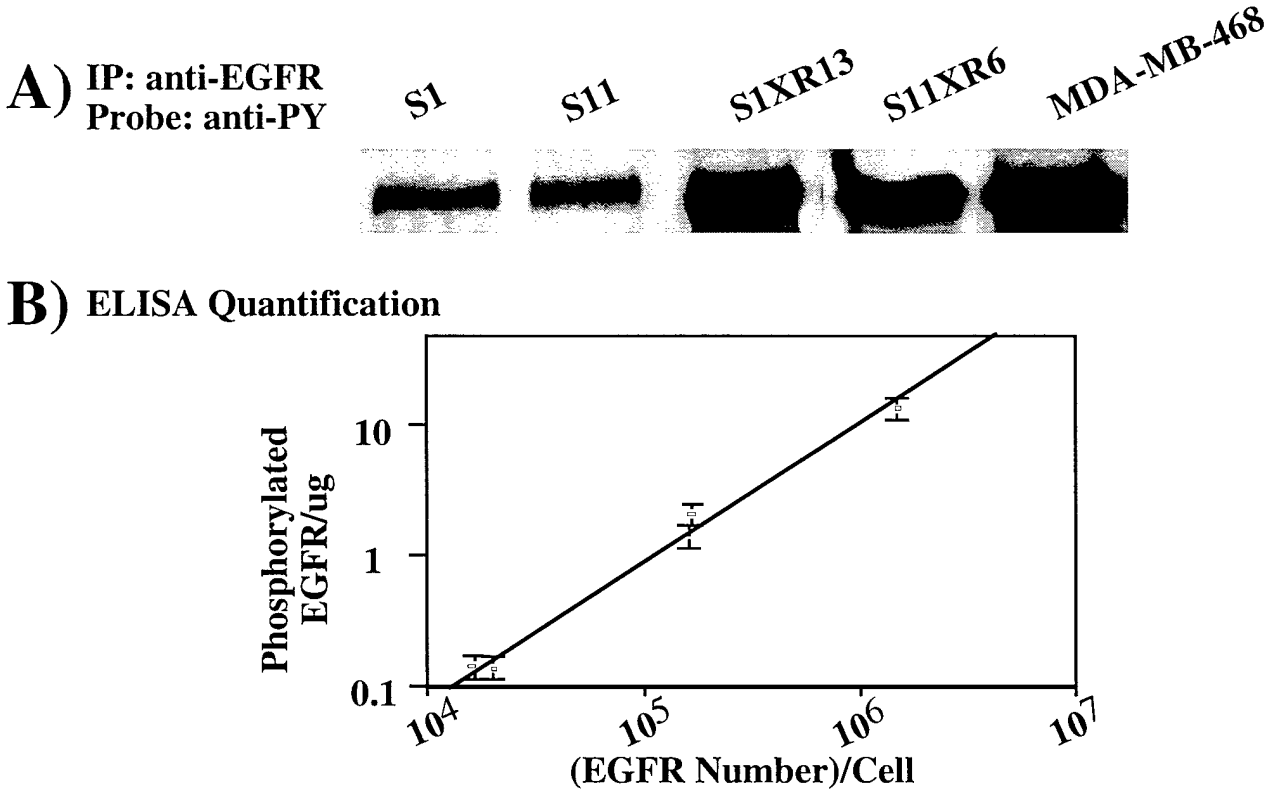


Figure 7: *Constitutive EGF-R associated PY increases linearly with the degree of receptor amplification.* **A)** EGF-R were immunoprecipitated from S1, S11, S1XR13, S11XR6 and MDA-MB-468 cells. Equal cellular protein concentrations of each cell type were separated by SDS-PAGE and PY was visualized by chemiluminesence of a Western blot with HRP conjugated recombinant PY antibody RC20. **B)** PY per cell was quantified by PY ELISA. EGF-R were isolated by cell lysis and captured by the monoclonal anti-EGF-R antibody 225. PY associated with the captured EGF-R was determined using the AP conjugated recombinant anti-PY antibody RC20. Level of PY per mg of cellular protein associated with the captured EGF-R in this ELISA was plotted against the number of EGF-R per cell.

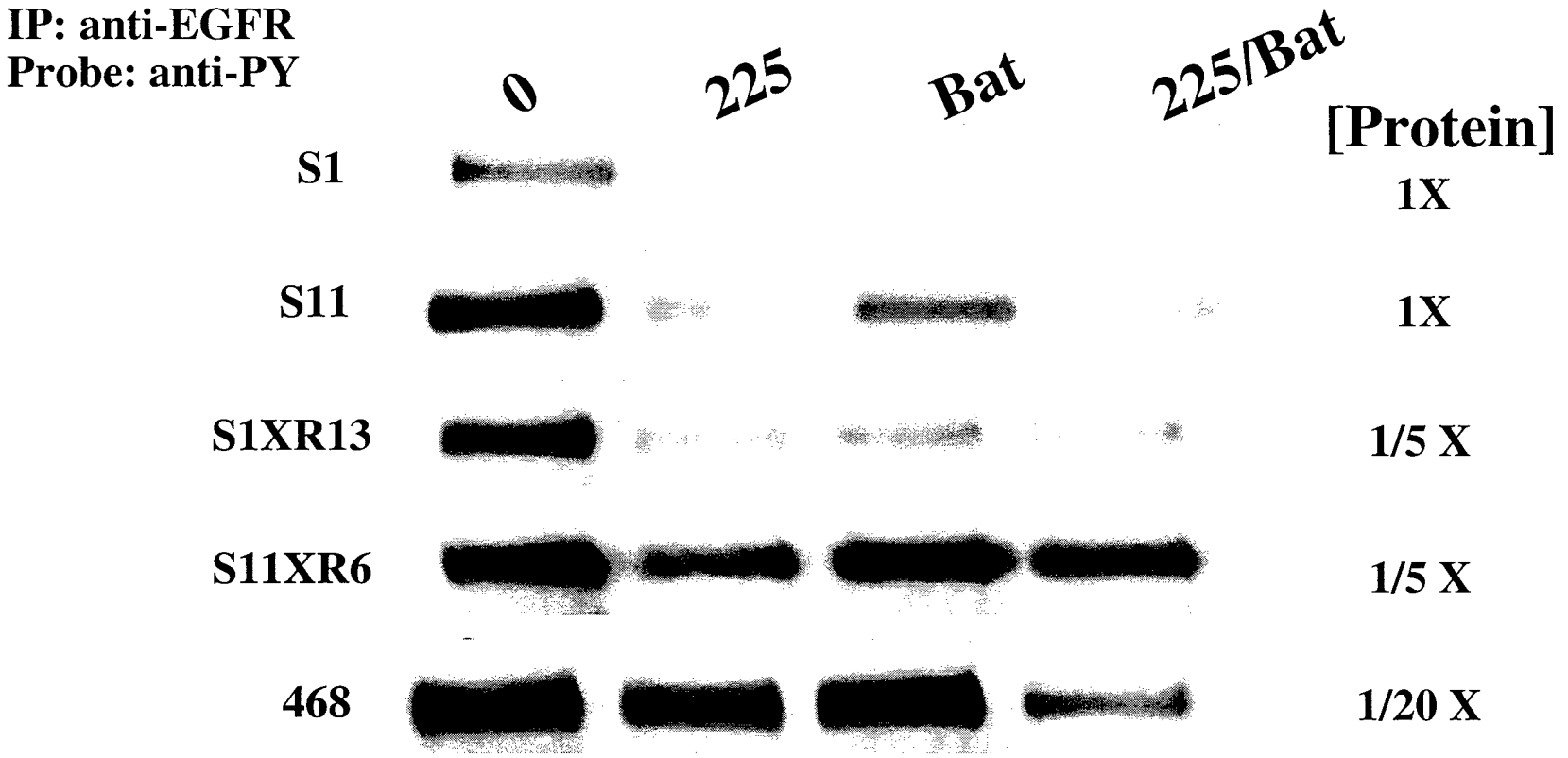


Figure 8: *Increased ability to capture ligand decreases the ability of antagonistic EGF-R antibodies and metalloprotease inhibitors to block constitutive EGF-R associated PY.* S1, S11, S1XR13, S11XR6 and MDA-MB-468 cells were treated with nothing (0), 5 mg/ml 225 (225), 10 mM Batimastat and 10 mM BB2116 (Bat), or simultaneously with 5 mg/ml 225, 10 mM Batimastat and 10 mM BB2116 (225/Bat) for 24 hours. EGF-R were then immunoprecipitated from all cells. S1 was diluted to 1.2 mg/ul, S11 was diluted to 1.5 mg/ul, S1XR13 was diluted to 0.25 mg/ul, S11XR6 was diluted to 0.3 mg/ul, and 468 was diluted to 0.08 mg/ul prior to immunoprecipitation. Equal volumes of each immunoprecipitation were separated by SDS-PAGE. **A)** PY was visualized by chemiluminesence of a Western blot with the HRP conjugated recombinant PY antibody RC20.

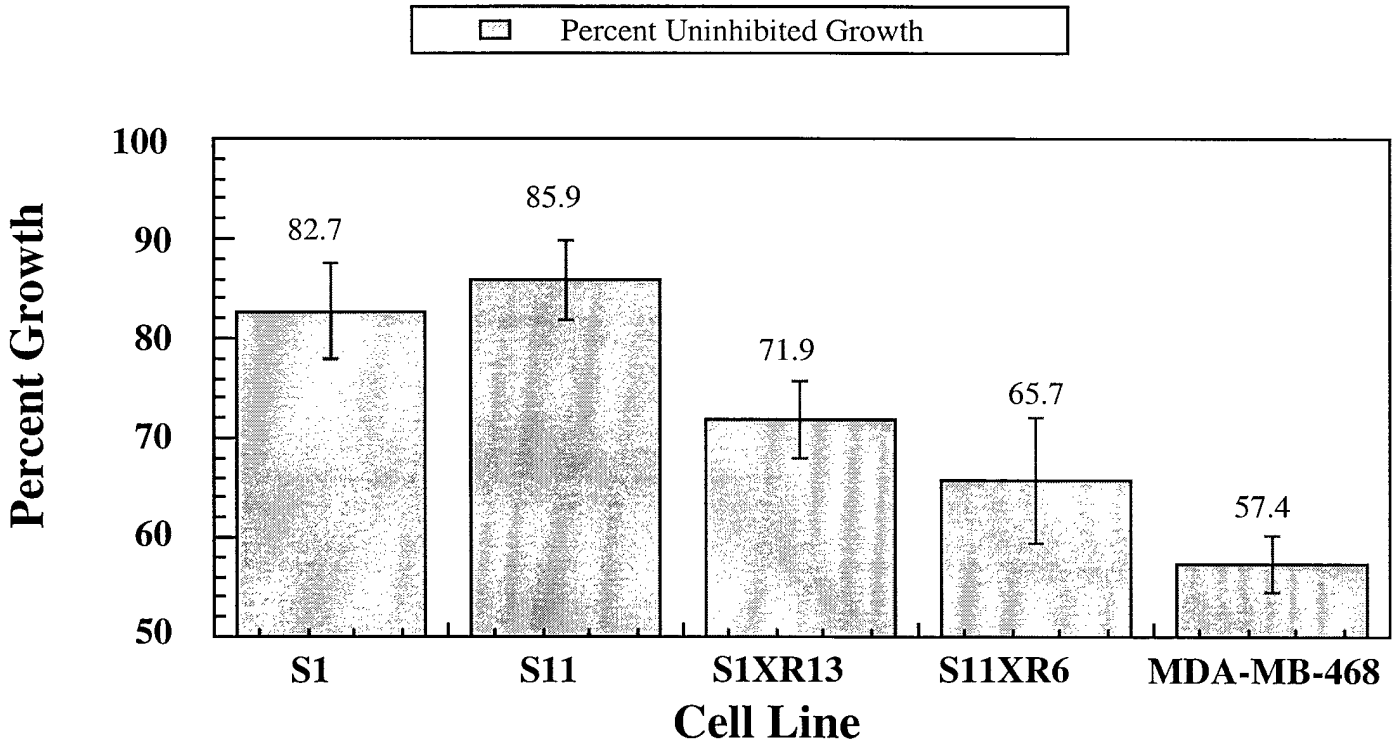


Figure 9: *Receptor amplified lines are more sensitive to the interruption of autocrine growth loops than unamplified lines.* S1, S11, S1XR13, S11XR6 and MDA-MB-468 were plated in low serum media for 24 hours at 37 C. Media was then changed to low serum media with or without antagonistic EGF-R antibody 225 and cells were grown for another 24 hours at 37 C. Cell numbers were determined by Coulter counting. Percent Uninhibited Growth = [Cell Number In 225/Cell Number In The Absence Of 225]x100. Numbers above bars are actual mean values.

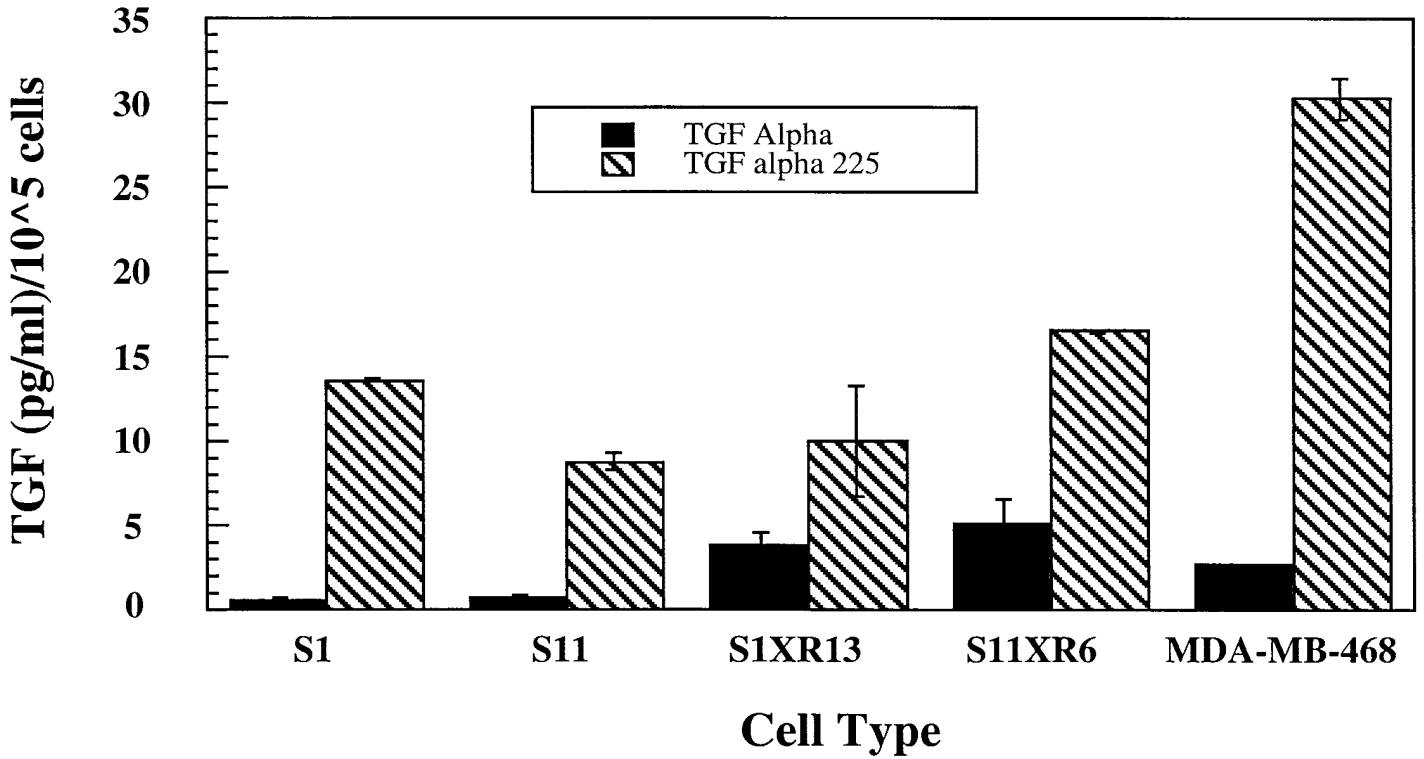
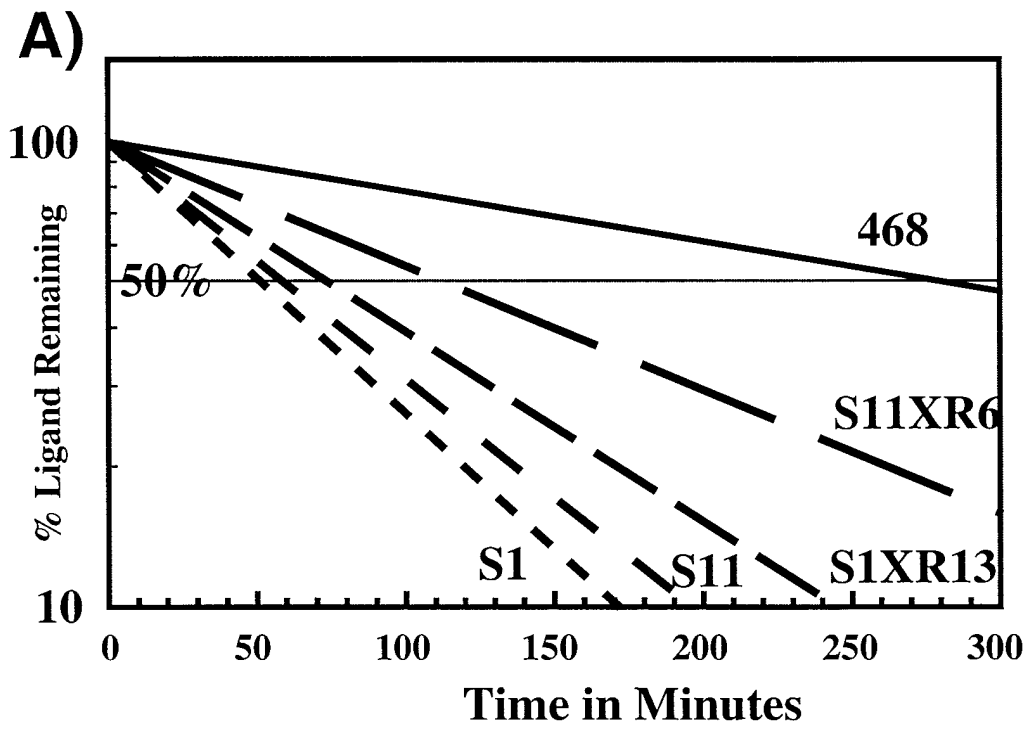


Figure 10: *Cells express equivalent levels of TGFα regardless of receptor number.* S1, S11, S1XR13, S11XR6 or MDA-MB-468 cells were treated with, or without 5 μg/ml antagonistic EGF-R antibody 225 for 24 hours. The concentration of TGFα excreted into the media by each cell type was then determined by RIA and normalized by cell number determined by Coulter counting. Solid bars show TGFα concentration in the media of cells not treated with antagonistic antibody. Diagonal striped bars show TGFα concentration in the media of cells treated with antagonistic antibody. Numbers above each bar are the actual TGFα concentration per 10^5 cells.



B)

Cell Type	$t_{1/2}$ (min)
S1	52
S11	60
S1XR13	74
S11XR6	112
468	278

Figure 11: *Receptor amplification prolongs the time that EGF remains associated with the cell prior to dissociation into the media.* S1, S11, S1XR13, S11XR6 and 468 cells were pulsed with 1 ng/ml 125I labeled EGF for 10 minutes. The cells were then chased with cold, ligand free media for 0, 3, 10, or 30 minutes at 37 C. At each time point, cells were solubleized in 2% SDS and counts associated with the solubleized material were determined by gamma counting. Ligand associated with the cell at 0 minutes post chase was defined as 100%. Percent ligand remaining versus time was plotted and best fit exponential lines through those points were extrapolated to the time at which 50% of original ligand CPM remain associated with the cell. **A)** Curve fit data. The 50% ligand remaining point is demarcated by the horizontal line. Time to reach the 50% ligand remaining is the intersection of the best fit line and the 50% ligand remaining line. **B)** Table of extrapolated t1/2 values for each cell line.

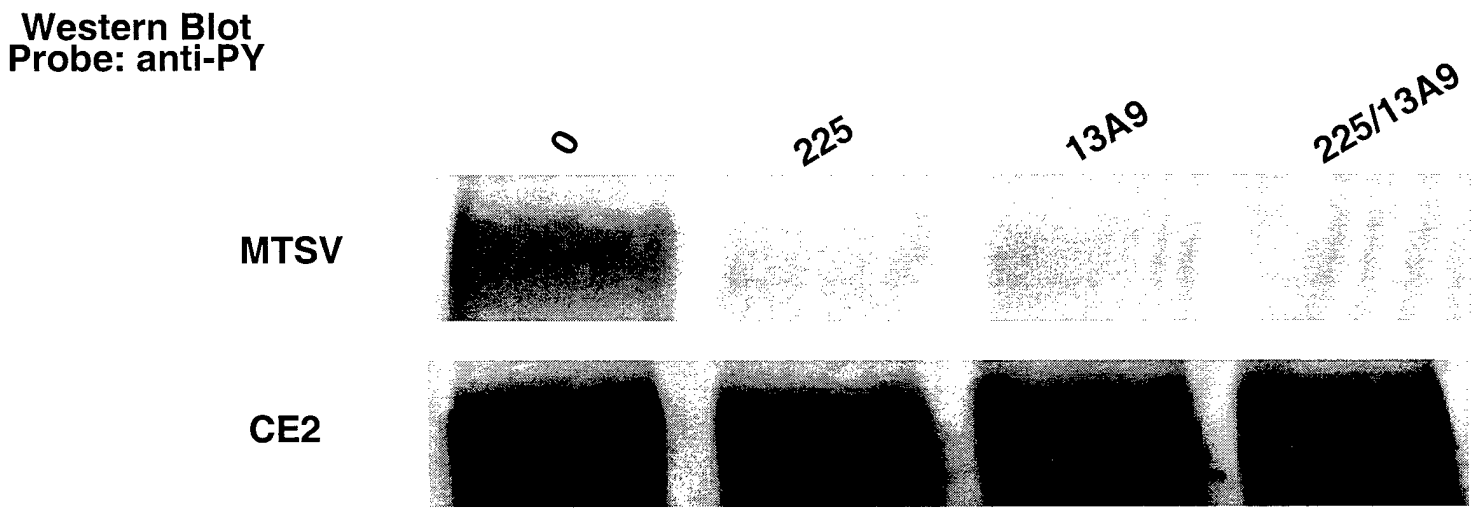


Figure 12: *Her2 amplification increases EGF-R associated constitutive PY in a ligand independent manner.* MTSV or CE2 cells were treated with nothing (0), 20 mg/ml antagonistic EGF-R antibody 225 (225), 20 mg/ml antagonistic EGF-R antibody 13A9 (13A9), simultaneously with 20 mg/ml 225 and 20 mg/ml 13A9 (225/13A9) for 30 minutes. EGF-R were then immunoprecipitated from all cells. Equal volumes of each immunoprecipitation were separated by SDS-PAGE. **A)** PY was visualized by chemiluminesence of a Western blot with the HRP conjugated recombinant PY antibody RC20.

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Ratiometric Assay of Epidermal Growth Factor Receptor Tyrosine Kinase Activation

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Activation of cells is frequently followed by tyrosine phosphorylation of proteins. To quantify this process, we developed a ratiometric enzyme-linked immunosorbent assay (ELISA) using epidermal growth factor receptors (EGFR) as a model. Microtiter dishes were coated with anti-EGFR monoclonal antibodies to capture the receptor followed by parallel detection of receptor and phosphotyrosine content with secondary antibodies. The ratio of these two parameters was found to directly reflect EGFR activation and was insensitive to the effect of receptor downregulation. Our assay could resolve differences in EGFR activation due to small changes (less than 1 ng/ml) in ligand. We found that phosphotyrosine detection by ELISA was 8- to 32-fold more sensitive than Western blot detection and could be reliably detected using as little as 4 ng of cellular lysate. Detection of EGFR levels by ELISA was 30 times more sensitive than Western blot analysis and was reliable for as low as 8 ng of cellular lysate per well. Because of the wide linear range of the ELISA, we could directly compare receptor activation in cell types with different EGFR expression levels. Our assay provides a rapid and sensitive method of determining EGFR activation status and could be easily modified to evaluate any tyrosine-phosphorylated protein. © 2000 Academic Press

Key Words: **epidermal growth factor receptor (EGFR); tyrosine kinase; enzyme-linked immunosorbent assay (ELISA); ratiometric.**

An important group of cell surface receptors possesses intrinsic tyrosine kinase activity. One prominent member of this group is the epidermal growth factor receptor (EGFR),[1] which is one of four members of a family of receptor tyrosine kinases (1). Ligand binding activates the intrinsic tyrosine kinase activity of the receptor leading to self-phosphorylation of its carboxy terminus (2). The phosphorylated receptor can then interact with other proteins and initiate signal transduction through proteins such as ras (3, 4). Activation and phosphorylation of the EGFR also induces endocytosis and sequestration of the receptors in endosomes (5–7). The endocytosed receptors can then be either recycled to the cell surface or degraded in lysosomes, resulting in receptor downregulation (5–7). In addition to downregulation, other spatial and covalent regulatory processes act upon the EGFR to attenuate its signaling (2).

The most proximal indication of tyrosine kinase receptor activation is self-phosphorylation. Virtually all biological effects attributable to the EGFR require kinase activity and receptor phosphorylation (8–12). This tyrosine phosphorylation has been well defined as the initiating event in several signal transduction pathways (13–15). In addition, increased tyrosine phosphorylation of the EGFR is a marker of poor prognosis in several cancers (11, 16–19).

Although the phosphorylation state of the EGFR is frequently used as an indicator of receptor activation, levels of receptor phosphorylation are rarely quantified. Western blot analysis is the most common method used to determine the approximate degree of EGFR phosphorylation (20, 21). Unfortunately, this method is not as sensitive as other methods, such as ELISA, and is not amenable to high-throughput assays. The limited linear range of film makes it very difficult to analyze samples differing greatly in EGFR expression level. Additionally, multiple blots must be run in parallel or the same blot must be stripped and reprobed

[1] Abbreviations used: EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; PBS, phosphate-buffered saline; HS, horse serum; PNP, *p*-nitrophenyl phosphate; NBCS, newborn calf serum; BSA, bovine serum albumin.

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with another antibody to determine both EGFR and phosphotyrosine levels. Assays that measure enzymatic activity toward exogenous substrates do not necessarily reflect the in situ activity of the EGFR (22–24).

Studies exploring the regulation of the EGFR and other tyrosine kinase receptors would be greatly facilitated by a rapid and sensitive assay of EGFR levels and tyrosine phosphorylation status. Unfortunately, the lack of practical methods for calibrating receptor and phosphotyrosine levels has been a substantial hurdle in the development of quantitative assays. However, for most studies the absolute level of receptor tyrosine phosphorylation is not as important as the relative levels. This is because most investigations examine changes in activity as a function of time and conditions. In these cases, an assay that quantified the ratio of phosphotyrosine to receptor mass would be sufficient. With this consideration in mind, we developed a very sensitive ratiometric ELISA for the quantification of phosphotyrosine, EGFR, and Tyr(P)/EGFR levels. A single 96-well plate can be used to simultaneously analyze four independent dilutions of both phosphotyrosine and EGFR for up to 12 separate samples. Phosphotyrosine can be detected at levels as low as 4 ng/well of protein extract. EGFR can be detected as low as 8 ng/well of protein extract. The linear range of phosphotyrosine detection by ELISA spans more than a 60-fold protein dilution range. The EGFR ELISA spans a protein range of 20-fold or more. The assay described in this report is sensitive and rapid and because it is internally normalized to EGFR, it is relatively insensitive to handling errors.

MATERIAL AND METHODS

Antibodies and Cells

Monoclonal anti-EGFR antibody 225 was purified from hybridomas obtained from the American Type Culture Collection (ATCC HB 8508; American Type Culture Collection, Rockville, MD) as previously described (25). Briefly, serum-free medium from hybridomas grown *in vitro* was collected and concentrated using an Amicon XM-50 filter. Antibodies were then purified by ammonium sulfate precipitation and by protein A–Sepharose Chromatography (25). Polyclonal rabbit anti-EGFR antiserum 448 was prepared by injection of EGFR isolated from A431 cell membranes (26) and boosted by injection of whole membranes using standard protocols (27). Alkaline phosphatase-conjugated goat anti-rabbit antibody was purchased from Sigma (St. Louis, MO). Horseradish peroxidase-conjugated goat anti-rabbit antibody was purchased from Pierce (Rockford, IL). Horseradish peroxidase or alkaline phosphatase-conjugated anti-Tyr(P) antibody RC20 was purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal EGFR antibody SC-03 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

MDA-MB-468, a human adenocarcinoma breast cell line, was purchased from the American Type Culture Collection (ATCC HTB 132; American Type Culture Collection). S1 and S11 variant cell lines were a generous gift from Dr. R. Buick. Both variant cell lines were isolated from the original MDA-MB-468 cell line (28, 29). The MDA-MB-468 cells and its variants were grown in L15 medium, supplemented with 20 mM sodium bicarbonate, 100 U/ml penicillin, 2.5 μg/ml streptomycin, 4 μM glutamine, 10% fetal bovine serum (FBS, Hyclone, Logan, UT). Eighteen hours prior to assay, cells were split into the medium containing only 0.5% FBS.

Tyr(P)/EGFR ELISA

All reagents are from Sigma unless otherwise noted. Flat-bottom high-capacity ELISA plates (Corning 25805-96, VWR Scientific Products, U.S.A.) were coated with 50 μl per well of 10 μg/ml monoclonal anti-EGFR mAb 225 in PBS/EDTA (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4, 0.6 mM EDTA) for 12 h at room temperature. Plates were then blocked with 200 μl per well of 10% horse serum (HS, Hyclone, Logan, UT) for 12 h at room temperature. Plates were stored at 4°C until use. For blocking and all subsequent steps, horse serum was passed through a 0.2-μm filter before use.

Cells were treated with 0–1.0 μg/ml recombinant human EGF (Peprotech Inc., Rocky Hill, NJ) for 10 min at 37°C. Cells were then placed on ice, aspirated, and rinsed once with 10 ml of ice-cold PBS/EDTA, 1 mM sodium orthovanadate. Cells were collected in 1 ml of ice-cold scraping buffer (PBS, 4 mM iodoacetate, 1 mM sodium orthovanadate, 1 μg/ml each of pepstatin, chymostatin, leupeptin, and aprotinin). After concentration by centrifugation at 1000*g* for 3 min, the scraping buffer was aspirated and the cell pellet was resuspended in 350 μl of ice-cold RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris–HCl, pH 7.2, supplemented with 4 mM iodoacetate, 1 mM orthovanadate, 1 μg/ml each pepstatin, chymostatin, leupeptin, and aprotinin). After lysis for 15 min on ice, insoluble material was removed by centrifugation at 18,500*g* at 4°C for 10 min.

Protein assays were performed in triplicate on each sample in 96-well assay plates (Corning 25880-96, VWR Scientific Products, U.S.A.) using the BCA protein assay kit (Pierce) as per manufacturer's instructions. All samples were brought to the same initial protein concentration and then diluted in 10% HS, 1 mM sodium orthovanadate.

Half of the mAb 225-coated ELISA plate was used for the phosphotyrosine ELISA and the other half was used for the corresponding EGFR ELISA. MDA-MB-468 protein lysate was added to the ELISA plate at concentrations of 1 ng to 8.1 μg/well for the phosphotyrosine and at 0.5 ng to 4.1 μg/well for the EGFR ELISA. S1 and S11 cell lysates were added to the ELISA plates at 0–20 μg/well for the phosphotyrosine ELISA and 0–10 μg/well for the EGFR ELISA. All samples were diluted in 10% HS, 1 mM sodium orthovanadate to a final volume of 50 μl/well and incubated on the ELISA plates at 37°C for 1 h.

Plates were aspirated and rinsed four times with a wash solution (300 mM NaCl, 20 mM Tris–HCl, pH 8.3, 0.1% sodium dodecyl sulfate, 0.05% NP-40). The alkaline phosphatase-conjugated anti-Tyr(P) antibody RC20 was diluted to 0.05 μg/ml in 10% HS, 1 mM sodium orthovanadate. The polyclonal anti-EGFR antiserum was diluted 1:1000 in 10% HS, 1 mM sodium orthovanadate. Fifty microliters of these diluted anti-Tyr(P) or anti-EGFR antibodies was added to each well of the respective halves of the ELISA plate and incubated for 2 h at room temperature.

The EGFR half of the ELISA plate was aspirated and rinsed four times with wash solution. Fifty microliters of alkaline phosphatase-conjugated goat anti-rabbit antibody diluted 1:6000 in 10% HS, 1 mM sodium orthovanadate was added to each of the anti-EGFR ELISA wells. The ELISA plates were again incubated for 2 h at room temperature.

Both halves of the ELISA plate were aspirated and rinsed four times with wash solution. Five-milligram *p*-nitrophenyl phosphate (PNP) tablets were dissolved to a final concentration of 1 mg/ml in reaction buffer (10 mM diethanolamine, 0.5 mM $MgCl_2$, pH 9.5), and 50 μl was added to each well of the ELISA plate. Absorbance of 405 nM was measured at 37°C at 11-s intervals over a 15-min period using the Molecular Devices Spectra MAX 250 ELISA plate reader and the Molecular Devices Soft MAX Pro (v. 1.2.0) acquisition software. Standard deviations and standard errors were calculated using Microsoft Excel.

Western Blotting

Protein samples were isolated as above described for the ELISA assay. Samples were diluted in water with 1 mM Na orthovanadate, reduced with 1% β-mercaptoethanol, 1% sodium dodecyl sulfate, and denatured for 10 min by boiling. The receptor was resolved by electrophoresis using a 7.5% gel and transferred to nitrocellulose using standard protocols (30). Phosphotyrosine was visualized with horseradish peroxidase-conjugated anti-Tyr(P) RC20 diluted to 0.05 μg/ml in 1% BSA blocking buffer (1% BSA, 10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20, 1 mM Na orthovanadate). The EGFR was visualized with polyclonal EGFR antibody SC-03 diluted to 0.08 μg/ml and a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody diluted 1:25,000. All bands were visualized by chemiluminesence (Renaissance, NEN, Boston, MA) using film and quantified by densitometry using the Bio-Rad Molecular Analyst software.

RESULTS AND DISCUSSION

The basic design of the assay is to capture the EGFR with a monoclonal antibody and then to detect Tyr(P) content and receptor levels in parallel with secondary antibodies. As a first test, we used MDA-MB-468 cells treated with or without EGF. The maximal increase in optical density per minute (ΔOD_{max}) for the colorimetric signal generated by the alkaline phosphatase detection enzyme was measured to ensure that the signal was proportional to conjugated enzyme levels. The sensitivity of our phosphotyrosine ELISA was determined following serial dilution of our cells. During the early phase of development for the assay, experiments were performed to determine duration of EGF treatment required to elicit maximal PY response (data not shown). Regardless of the cell types used, PY signal was maximal by 2 min posttreatment. On rare occasions, the 10-min time point was slightly greater than the 2-min time point. A 10-min treatment time was used in all subsequent experiments.

The data that are shown in Figs. 1, 3, and 5 are representative sets, each from a single assay performed on the same day. Intraassay variability (replicates of the same protein concentration, from the same lysis performed at the same time) was small and not statistically significant. Interassay variability (numerical values for the same protein concentration, from the same cell type treated the same way but performed on different days) was never more than twofold. We attribute these differences to parameters that are difficult, if not impossible, to control: For example, changes in serum lot, fraction of cells in different phases of the cell cycle, and different ligand lots.

As shown in Fig. 1, with EGF treatment, the phosphotyrosine detection threshold was 4 ng of total cell protein. In contrast, the minimal detectable threshold for untreated cells was approximately 16 ng total cellular lysate. The signal in untreated cells is due to a low level of autocrine growth factor production (31, 32). Nonspecific phosphotyrosine background was elevated when bovine serum albumin was used as a blocking agent instead of 10% horse serum (data not shown).

As the amount of cell extract was increased, phosphotyrosine levels increased linearly for both EGF-stimulated and unstimulated cells up to a total of 1 μg of protein. Thus, with EGF treatment, phosphotyrosine could be detected over a 250-fold dilution range. In the

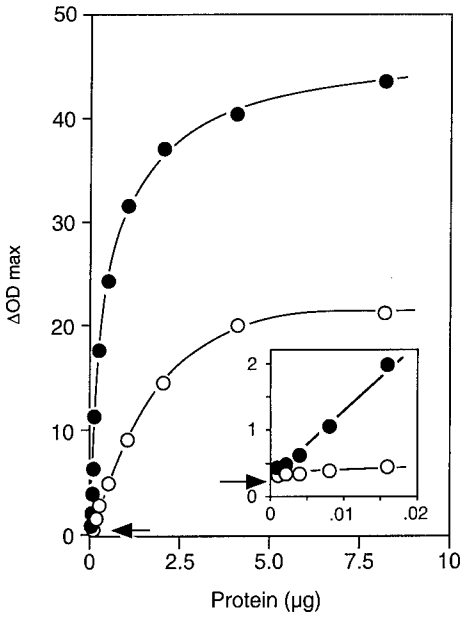

FIG. 1. ELISA analysis of phosphotyrosine levels. MDA-MB-468 cells were incubated with or without 100 ng/ml EGF for 10 min at 37°C. The ΔOD_{max} of PY was determined by ELISA using a kinetic read over a 15-min time course. Inset is a magnification of the region from 0 to 0.02 μg of protein. Open circles are MDA-MB-468 cells without EGF treatment. Filled circles are MDA-MB-468 cells with 100 ng/ml EGF treatment. Arrows are the values of blank wells. Data are representative of a typical experiment.

absence of EGF treatment, phosphotyrosine could only be detected over a 64-fold dilution range, due to the higher detection threshold. The curves plateau at different ΔOD_{max} levels because the capture antibody is only binding low phosphotyrosine content EGFR without EGF treatment, while the antibody is binding high phosphotyrosine content EGFR with EGF treatment. The addition of phenyl phosphate completely eliminated all Tyr(P) signal, demonstrating the specificity of our antibodies for Tyr(P) (data not shown).

Different assay conditions were explored to maximize the signal/noise ratio. We tried different blocking agents, wash conditions, extraction conditions, and antibodies. Three separate blocking agents, HS, newborn calf serum (NBCS), and BSA, were tried. We found that 10% NBCS displayed the highest background signal level and the 2.5% BSA background signal was only slightly lower. In contrast, 10% HS displayed a far lower background signal compared to either NBCS or BSA, and was used in all subsequent experiments. Decreasing the absolute concentration of either serum or BSA increased the background signal, apparently due to an increased level of nonspecific binding. Very little effect was observed by increasing the absolute concentration of the blocking agents. A less-stringent wash (PBS/azide/0.5% Tween 20) slightly elevated the background signal, but had no effect on overall signal.

It should be noted that although inclusion of phosphatase inhibitors increased the stability of receptor-associated Tyr(P), their levels did decrease with time. For example, following incubation overnight at 4°C, we found that approximately 30% of the Tyr(P) content was lost from all samples.

The use of different antibodies was also explored to maximize assay sensitivity and specificity. We found that use of the recombinant RC20 anti-Tyr(P) antibody reduced background noise and enhanced signal above that seen for polyclonal anti-Tyr(P) antibodies. Higher dilutions of RC20 had little effect on background noise, but slightly reduced the specific signal. In contrast to RC20, it was necessary to carefully optimize the concentration of polyclonal anti-EGFR or anti-Tyr(P) antibodies to obtain the best signal-to-noise ratios. We compared a polyclonal anti-EGFR antibody generated in our laboratory (ab448) to the commercially available polyclonal antibody SC-03 (Santa Cruz Biotechnology, Inc.). We found that SC-03 exhibited a nearly identical dynamic range and detection limit as ab448 when used at a fourfold higher concentration (1:250 versus 1:1000; data not shown). The SC-03 antibody exhibited a lower background signal than our antibody, probably because it is purified.

The detection ranges of the ELISA were directly compared to those obtained by Western blot analysis. As shown in Fig. 2, a minimum of 30–60 ng/lane of total protein was required for phosphotyrosine detection by Western blot following cell stimulation with EGF. In the absence of EGF treatment, the minimum protein concentration required for phosphotyrosine detection was approximately 0.5 μg/lane. The phosphotyrosine ELISA is therefore at least 30-fold more sensitive than Western blotting in the absence of EGF and 8- to 16-fold more sensitive in the presence of EGF.

Figure 2 shows that, without EGF, the levels of phosphotyrosine quantified by densitometry increased fairly linearly up to 4 μg of total protein. Because the film only has a dynamic range spanning approximately 8-fold of protein dilutions, the user must change exposure times depending on whether high or low protein concentrations are to be quantified. With 100 ng/ml EGF treatment, the phosphotyrosine signal increases from 30 to approximately 260 ng/well of total protein. As in the cells without EGF treatment, the dynamic range of the film remains approximately 8-fold and the addition of EGF simply shifts the detection limit leftward to a lower protein concentration. The dynamic range of phosphotyrosine detection by ELISA is therefore 8- to 32-fold greater than Western blot analysis. The range of quantification by densitometry is largely dictated by the dynamic range of the film, so different dilutions can be analyzed by altering exposure times. However, because the required exposure time changes with protein concentration and absolute phosphoty-

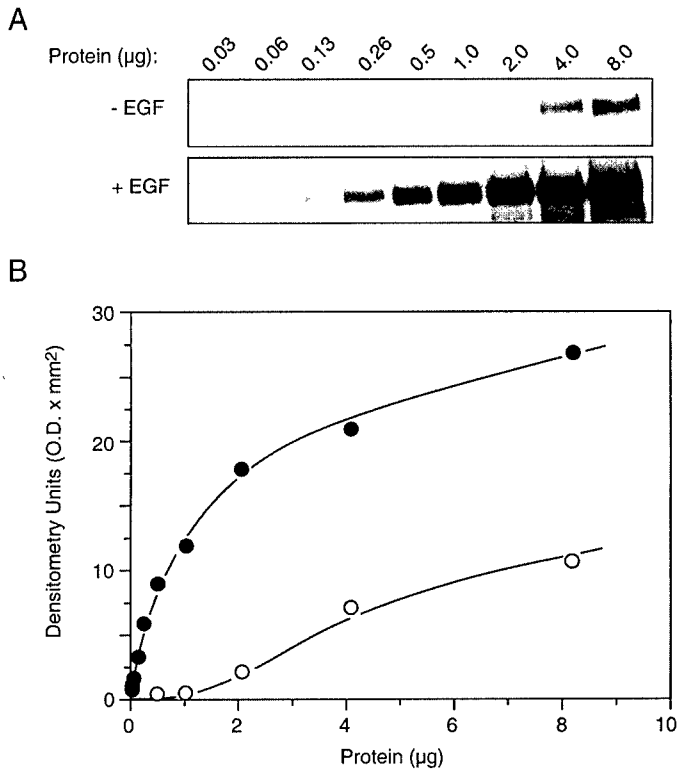

FIG. 2. Western blots and densitometric analysis of Tyr(P) levels. MDA-MB-468 cells were incubated with or without 100 ng/ml EGF for 10 min at 37°C. (A) Tyr(P) was visualized by HRP-conjugated anti-Tyr(P) antibody RC20 and chemiluminescence. (B) Densitometry for blot in A is graphed as a function of protein concentration. Open circles are MDA-MB-468 cells with no EGF treatment. Filled circles are MDA-MB-468 cells with 100 ng/ml EGF treatment. Data are representative of a typical experiment.

rosine levels, it is very difficult to quantify both treated and untreated cells on the same film by densitometry.

We also determined the detection limits for the EGFR in parallel with the phosphotyrosine detection limits. As shown in the inset to Fig. 3, EGFR can be detected by ELISA at lysate concentrations as low as 8 ng/well. The data in Fig. 3 also show that the upper limit of EGFR detection is approximately 0.5 μg of cell lysate. Thus, the ELISA displays at least a 60-fold detection range for the EGFR. As expected, the absolute range of EGFR detection was identical regardless of whether or not the cells were stimulated with EGF. The identical upper plateau values show that the absolute receptor assay range is limited by the binding capacity of the ELISA plates for the capture antibody. In addition, the lack of difference between the levels of EGFR in cells treated either with or without EGF shows that the degree of EGFR phosphorylation does not affect EGFR detection by our secondary anti-EGFR antibodies.

We compared the results from the EGFR ELISA to Western blots. As shown in Fig. 4, the lower limit of EGFR detection by Western blotting was approximately 0.25 μg, or about 30-fold less sensitive than the EGFR ELISA. Figure 4 also shows that, although there is a faint band at 130 ng protein (Fig. 4A), this sample did not have sufficient signal intensity to be distinguished from the background by the densitometer and is therefore not included in the quantification (Fig. 4B). Western blots were roughly linear for EGFR detection up to 2 μg/lane of protein. Thus, the dynamic range of EGFR quantification by Western blot followed by densitometry was approximately 8-fold and encompasses the entire dynamic range of film.

We compared the linear range of the phosphotyrosine and EGFR ELISAs as defined by an r^2 value greater than 0.90. Figure 5A shows the linear region for the phosphotyrosine ELISA that displays a slope of 8.9 without EGF and 84 with the addition of EGF. This gives a 9.4-fold induction of phosphotyrosine with EGF treatment. The slopes encompassing the linear portion of the phosphotyrosine curve by Western blot analysis yielded a phosphotyrosine induction of 10.4-fold with EGF stimulation (Fig. 2) which is in good agreement with the ELISA data. The linear regions of the curves for EGFR quantification by ELISA are shown in Fig. 5B. The slopes of the curves were 71 without EGF and 73 with the addition of EGF.

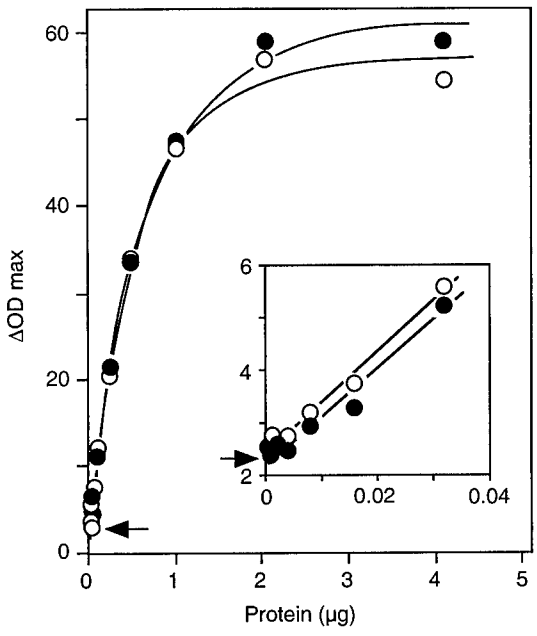

FIG. 3. ELISA analysis of EGF-R levels. MDA-MB-468 cells were incubated with or without 100 ng/ml EGF for 10 min at 37°C. ΔOD_{max} of EGF-R was determined by ELISA using a kinetic read over a 15-min time course. Inset is a magnification of the region from 0 to 0.04 μg of protein. Open circles are MDA-MB-468 cells without EGF treatment. Filled circles are MDA-MB-468 cells with 100 ng/ml EGF treatment. Arrows are the values of blank wells. Data are representative of a typical experiment.

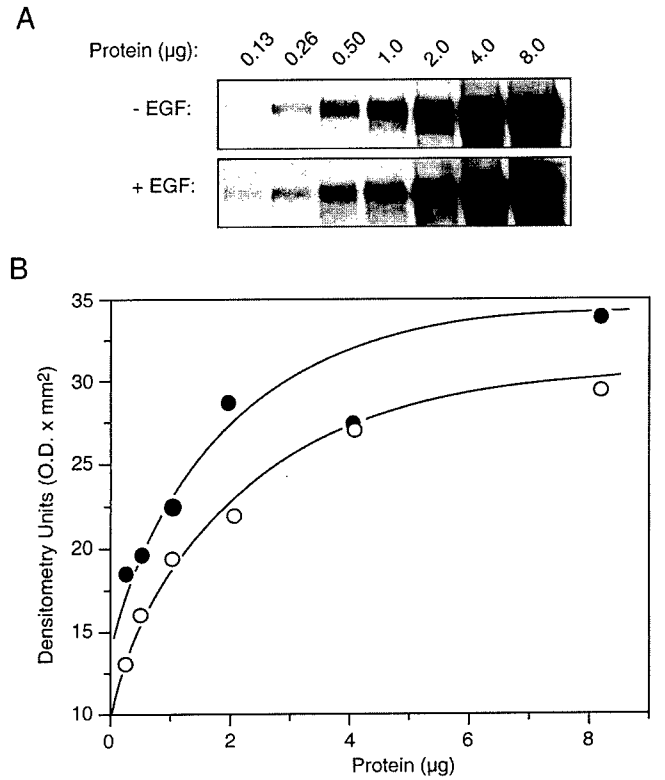

FIG. 4. Western blots and densitometric analysis of EGFR levels. MDA-MB-468 cells were incubated with or without 100 ng/ml EGF for 10 min at 37°C. (A) EGFR was visualized by polyclonal anti-EGFR primary antiserum SC-03 and HRP-conjugated goat anti-rabbit secondary antibody using chemiluminescence. (B) Densitometry for blot in A is graphed as a function of protein concentration. Open circles are MDA-MB-468 cells without EGF treatment. Filled circles are MDA-MB-468 cells with 100 ng/ml EGF treatment. Data are representative of a typical experiment. The two lines are parallel, but shifted due to differences in film background between the two blots.

The number of EGFR per cell will dictate the range of protein over which the ELISA assay is linear. Our results have suggested that, for similarly sized cells, the linear range of EGFR is approximately one-half of the phosphotyrosine linear range, indicating that the EGFR ELISA has approximately one-half the dynamic range (data not shown). This is most likely due to the use of polyclonal antibodies and a conjugated polyclonal secondary antibody for detection in the EGFR ELISA. The phosphotyrosine ELISA, on the other hand, uses a directly conjugated monoclonal for detection. Polyclonal antibodies for phosphotyrosine were initially used in our assay but were less suitable than the high-affinity recombinant antibody RC20. The higher affinity of the recombinant antibody is probably responsible for an observed decrease in background noise and an increase in signal intensity, leading to a better assay dynamic range.

As shown in Fig. 1, the phosphotyrosine ELISA is generally linear from 0 to 1 μg/well of lysate for cells expressing 10^6 EGFR per cell. For cells with 10^5 EGFR per cell, the phosphotyrosine assay is linear from 0 to 5 μg/well (data not shown). Cells with approximately 10^4 EGFR per cell have a linear phosphotyrosine assay from 0 up to approximately 20 μg/well (data not shown). The EGFR linear ranges are approximately half the concentrations used for phosphotyrosine detection.

The ratio of Tyr(P)/EGFR should reflect the degree of EGFR activation within cells. Therefore increasing ligand concentrations should result in a proportional increase in Tyr(P)/EGFR levels until all EGFR are saturated with ligand and the cell is maximally activated. Our assay demonstrates this supposition as shown in Fig. 6. The MDA-MB-468 cells express 1.5×10^6 EGFR per cell, whereas the S1 and S11 cells have

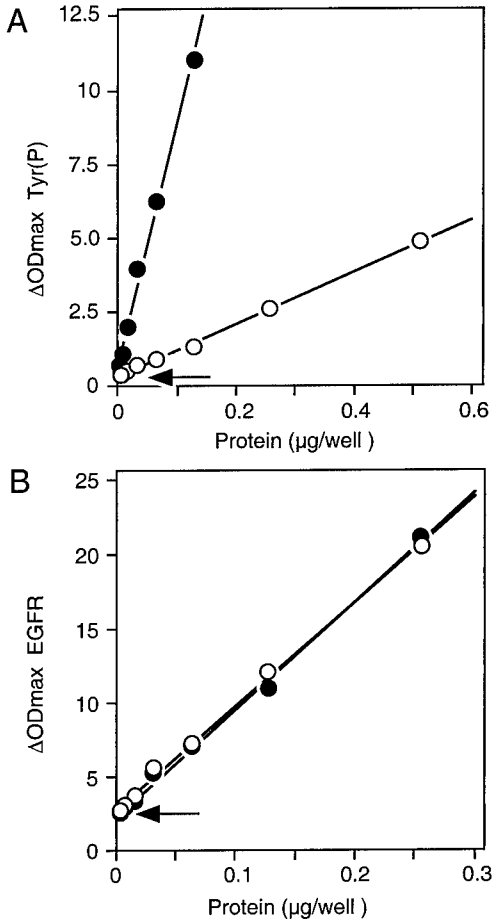

FIG. 5. Linear range of the ELISA assays for either Tyr(P) or EGFR. (A) Linear region of Tyr(P) ELISA graph shown in Fig. 1. (B) Linear region of EGF-R ELISA graph depicted in Fig. 3. Open circles are MDA-MB-468 cells without EGF treatment. Filled circles are MDA-MB-468 cells with 100 ng/ml EGF treatment. Arrows indicate the sample blank values.

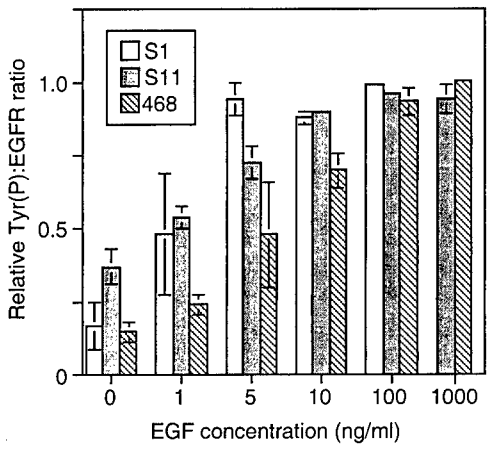

FIG. 6. Percentage maximal Tyr(P)/EGFR ratio as a function of EGF concentration in ng/ml. MDA-MB-468, S1 variant, or S11 variant cells were stimulated with 0, 1, 5, 10, 100, or 1000 ng/ml EGF for 10 min at 37°C. Tyr(P) and EGF-R ELISAs were performed for each cell type at each ligand concentration. The slopes PY ΔOD_{max} or EGF-R ΔOD_{max} versus μg protein were used to calculate Tyr(P): EGFR. Samples were normalized for percentage maximal Tyr(P)/EGFR. Error bars are the standard error of the mean based on 2–4 independent experiments.

1.6×10^4 and 6.6×10^4 EGFR per cell, respectively (28, 29). The MDA-MB-468 cell line and the S variants, S1 and S11, were treated with increasing concentrations of EGF for 10 min. Proteins were then extracted and the activation status of the EGFR was analyzed using our ratiometric assay. As the EGF concentration was increased, the ratio of Tyr(P)/EGFR also increased. As expected, the MDA-MB-468 cells required a higher concentration of EGF to achieve a given degree of receptor activation due to their higher level of receptor expression. When a ligand concentration sufficient to saturate all receptors was used, the Tyr(P)/EGFR was maximal and identical for all cell lines (data not shown). The range of receptor expression exhibited by MDA-MB-468 cells and variants spans the range reported for EGFR expression in a variety of different cell types. Therefore, our ability to successfully quantify receptor activity in these cells indicates that our ratiometric assay should be useful for all types of EGFR-expressing cells.

The ratio of Tyr(P)/EGFR gives a measure of receptor activation with respect to receptor levels. As EGFR are degraded during downregulation, those receptors will no longer be captured in our assay so the Tyr(P) associated with that receptor will also be lost. Because of this, if receptor Tyr(P) is only removed by receptor degradation during downregulation, Tyr(P)/EGFR ratios will remain constant. Any decrease in Tyr(P)/EGFR should reflect a loss in phosphotyrosine from the EGFR.

Adapting this ratiometric assay for the use with other receptors or signaling molecules should be relatively simple. A monoclonal antibody to the protein under analysis is used as a capture antibody. A second, polyclonal, antibody is then required for detection of the captured protein. The Tyr(P) antibody and goat anti-rabbit AP-conjugated antibodies used in our assay would also be used in an ELISA designed for use with another protein. The use of 10% horse serum and all of our assay conditions should be equally applicable. The Tyr(P):protein ratio should provide the same activation status information and be equally insensitive to handling errors for all molecules.

Our ratiometric Tyr(P)/EGFR assay is a rapid, sensitive, and reliable means to assess EGFR activity in all cell types. Because the assay is conducted in a 96-well format, several protein dilutions can be used for each sample increasing the accuracy of the final reading. The data obtained on a single ELISA plate would require 5–10 standard size Western blots to achieve the same accuracy. The change in EGFR activity induced by small changes in ligand concentration (less than 1 ng/well) can be readily detected and compared between different cell types in our assay. The activation readout, Tyr(P)/EGFR, is insensitive to receptor downregulation and is an indirect readout of receptor dephosphorylation. This assay should allow future studies of receptor–ligand interactions to be performed faster and with increased accuracy.

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